Synopsis of the proposed work plan
Title of research proposal:

Studies on optimization, Characterization & Scale up of Laccase from Thermophilic bacteria

Submitted for the degree of
Doctor of philosophy in
Microbiology
Faculty of science and Technology

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Dec-2020

Introduction:

Extremophiles are organisms that have been discovered on earth that survive in environments that were once thought not to be able to sustain life. These extreme environments include intense heat, highly acidic environments, extreme pressure and extreme cold. Extremophilic organisms are primarily prokaryotic (archaea and bacteria), with few eukaryotic examples. alkaliphilic (optimal growth above pH 9); halophilic (optimal growth in environments with high concentrations of salt); thermophilic (optimal growth between 60 and 80 °C [140 and 176 °F]); hyperthermophilic (optimal growth above 80 °C [176 °F]).

Different organisms have developed varying ways of adapting to these environments. Which is due to the unique enzymes used by these organisms, called "extremozymes," enable these organisms to function in such forbidding environments. These creatures hold great promise for genetically based medications and industrial chemicals and processes.

Thermophiles are a group of heat loving microbes thriving at high temperature usually more than 45°C. They are inhabitants of various ecological niches like deep sea hydrothermal vents, terrestrial hot springs, and other extreme geographical/geological sites including volcanic sites, tectonically active faults as well as decaying matters such as the compost and deep organic landfills. All thermophiles require a hot water environment, but some thrive in more than one extreme, such as those with high levels of sulfur or calcium carbonate, acidic water, or alkaline springs. Regardless of varying environmental conditions, the ability of thermophiles to thrive in extremely hot environments lies in extremozymes, enzymes geared to work in extremely high temperatures. The amino acids of these extremozymes have special tricks to retain their twisted and folded 3D structures in high heat.

Alkalophiles are the microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near-neutral pH value of 6.5 The cell surface may play a key role in keeping the intracellular pH value in the range between 7 and 8.5, allowing alkaliphiles to thrive in alkaline environments Alkaliphiles have made a great impact in industrial applications. Such organisms are to be found in soils, marine sediments, and high Ca^{2+} ground waters. Dilute soda lakes yield very diverse populations of such alkaliphiles at least when freshly sampled.

Halophiles are salt-loving organisms that inhabit saline and hypersaline environments. Halophiles may be classified as slight, moderate or extreme, and as obligate halophiles or halotolerant. Halophiles and their biomolecules are useful for applications in biotechnology, medicine and industry.

Haloalkaliphiles are mostly confined to very hypersaline soda lakes and soda deserts. To date, the majority of isolates from such sites have been haloalkaliphilic Archaea, although some Bacillus types are also known.

Enzyme is a substance that acts as a catalyst in living organisms, regulating the rate at which chemical reactions proceed without itself being altered in the process. Substrates are the substances on which enzymes act. The conventional way of discovering enzymes is by growing organisms and then isolating enzymes in some way from parts of plants, animal organs, inside of microorganisms, or in their immediate environment. Greatest variation in physical properties is found in the enzymes of microorganisms. Temperature affects enzyme activity, increasing it to reach a peak, which corresponds to the optimal enzyme activity. Beyond this maximum, enzyme

activity rapidly drops. The optimal temperature for enzymes in different organisms is different. The inactivating effect of temperatures above optimum temperature is due to protein denaturation. pH affects enzyme activity, by influencing the state of dissociation of functional groups involved in the ES complex. Enzymes have an optimum pH and extreme values of pH cause enzyme denaturation.

The ability of thermophilic enzymes to work at high temperatures implies many advantages for their applications in industrial reactors (or fermenters). All of these advantages, resumed in are directly derived from the exceptional stability of these proteins: i) at high temperature, the viscosity of culture broth decreases, increasing the mass transfer and making easy the mixing and pumping of the substrates. This advantage is extremely important in some of the present applications of the enzymes, as for the hydrolysis of polysaccharides Sterility requirements are not as stringent. Are more resistant than mesophilic counterparts to denaturing agents (Organic solvents, high and low pH) often present in the reactions finally, as many high scale fermentation processes generate heat, the common and expensive cooling of reactors could be avoided if thermophiles or their enzymes were used.

Halophilic (salt loving) microorganisms have evolved physiological and genetic characteristics for success in their harsh saline environments. These characteristics include internal compatible solutes, efficient ion pumps, UV absorbing pigments, and acidic proteins, which help them to resist osmotic stress and the denaturing effects of salts, as well as the accompanying intense solar radiation. Currently established applications of halophiles are limited primarily to compatible solutes in the cosmetic industry, B carotene and hydrolyses in the nutritional and food industries. They include specialized applications for stable enzymes from halophiles for use as industrial catalysts, some capable of functioning in organic solvents. Metabolic activities of halophiles are also being tapped for a variety of environmental applications, such as bioremediation of pollutants in saline wastewaters. Some subcellular components, such as polyhydroxyalkanoate granules, gas vesicles, and phospholipid vesicles, and bioactive compounds like halocins are being developed for medical applications. Applications of halophilic microorganisms are varied and represent significant commercial opportunities in the chemical, environmental, biofuel, medical, and educational industries.

Alkalophilic enzymes, especially extracellular enzymes, are able to function in their catalytic activities under high alkaline pH values because of their stability under these conditions. . Alkaline enzymes often show activities in a broad pH range, thermostability, and tolerance to oxidants compared to neutral enzymes.

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases (PPO) that catalyze the oxidation of various substituted phenolic compounds by using molecular oxygen as the electron acceptor. The ability of laccases to act on a wide range of substrates makes them highly useful biocatalysts for various biotechnological applications.Laccases are the most numerous members of the multicopper protein family, which also includes tyrosinases, monoxygenases, and dioxygenases. Phylogenetically, these enzymes have developed from small sized prokaryotic azurins to eukaryotic plasma proteins ceruloplasmin (Claus 2003). They contain four histidine-rich copper-binding domains, which coordinate the types 1–3 copper atoms that differ in their environment and spectroscopic properties (Messerschmidt and Huber

1990) The first laccase was reported in 1883 from Rhus vernicifera, the Japanese lacquer tree (Reinhammar 1984), from which the designation laccase was derived. Despite the knowledge about the widespread occurrence of prokaryotic laccases, until now, the complete purification and characterization of only three bacterial laccases have been achieved so far. The first report of prokaryotic laccase is from the rhizospheric bacterium Azospirillum lipoferum (Givaudan et al. 1993), where laccase occurs as a multimeric enzyme composed of a catalytic subunit and one or two large chains. The enzyme plays a role in cell pigmentation and utilization of plant phenolic compounds (Faure et al. 1994, 1995) and/ or electron transport (Alexandre et al. 1999).

Review of literature:

Rajput & Mishra(2018) studied on Biodegradation of Pyridine Raffinate using Bacterial Laccase isolated from Garden Soil. Laccase was isolated from the bacterium *Pseudomonas fluorescens* and found to be an effective method to biodeteriorate pyridine raffinate instead of using harmful chemicals.It was observed that O.D. was reduced (max. 0.2327 at 501 nm) measured by UV/VIS Spectrophotometer taking raffinate as a reference.It signifies the oxidation of compounds present in the effluent that leads to the reduction in C.O.D. And found to be an effective method for the treatment of effluent.

Wang et.al(2019) studied on Characterization and application of a novel laccase derived from Bacillus amyloliquefaciens and exhibited that laccases demonstrate a promising potential in various environmental and industrial applications. a bacterial strain isolated from soil exhibited the laccase activity,which was subsequently characterized and named as Bacillus amyloliquefaciens TCCC 111018. recombinanat laccase gene rLACwould be an ideal candidate for lots of biotechnological and industrial applications due to its stability in the extreme conditions, including but not limit to pH, high temperature, halides, heavy metals and detergents.

Shafiei et al (2019) studied on Cohnella sp. A01 laccase: thermostable, detergent resistant, anti-environmental and industrial pollutants enzyme and exhibited that Laccase (EC 1.10.3.2; benzenediol; oxygen oxidoreductases) is a multi-copper oxidase that catalyzes the oxidation of phenols, polyphenols, aromatic amines, and different non-phenolic substrates with concomitant reduction of O2 to H2O.The remaining activity and structural properties of Cohnella sp. A01 laccase in extreme conditions such as high temperatures and presence of metals, detergents, and organic solvents suggest the potential of this enzyme in biotechnological

and industrial applications.This process has been patented in Iranian Intellectual Property Centre under License No: 91325.

Claus (2003) studied on structure, reactions, distribution of Laccases and exhibited that Laccases (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductases) are multi-copper proteins that use molecular oxygen to oxidize various aromatic and non-aromatic compounds by a radical catalyzed reaction mechanism.The enzymes are involved in the pathogenicity, immunity and morphogenesis of organisms and in the metabolic turnover of complex organic substances such as lignin or humic matter Phylogenetically, laccases are members of the multi-copper protein family including ascorbate oxidase, ceruloplasmin and bilirubin oxidase.

Neifar et al(2018) studied on Modeling and Optimization of Textile Dye Decolourization Using Laccase-Producing, Alkali-Halotolerant Bacteria and isolated new alkali-halotolerant bacteria from Tunisian desert and to select efficient strains for degradation of textile azo dyes, Bezactiv Blue S-Matrix 150 and Tubantin Brown GGL. Out of 50 collected samples 16 showed a laccase activity. Phylogenetic analysis indicated that the best laccase-producing and dye-degrading strain was affiliated with the genus *Pseudomonas*.

Chandra & Chowdhary studied on Properties of Bacterial Laccases and Their Application for Bioremediation of Industrial Wastes. In this review the bacterial laccase has been focused on the bioremediation of various industrial pollutants. A brief description on structural molecular and physicochemical properties has been done. Besides, the mechanism by which reaction is catalyzed, physical basis of thermostability and, enantioselectivity which requires more attention for researcher, and laccase application invarious fields of biotechnology has been pointed out.

Siroosi et al(2016) studied on Purification and characterization of an alkaline chloride tolerant laccase from a halotolerant bacterium, Bacillus sp. strain WT and exhibited that laccase oxidize different aro-matic or inorganic substrates. In present work, different bacterial strains isolated from Urmia lake, ahypersaline lake in northwest of Iran, were screened to find laccase-producing ones. Spore and an extra-cellular enzyme from a halotolerant spore-forming bacterium, Bacillus sp. strain WT The extracellular laccase (0.01 U mL−1) decolorized sulphonyl green BLE up to 97% at pH 7.0after two h incubation at 35◦C, without any addition of mediators

Turner et al. (2007) Studied on Potential and utilization of thermophiles and use of thermostable enzymes in biorefining. During study he focused on conversion of carbohydrate containing raw materials by thermophilic enzymes. Their importance in biorefineries is explained using examples of lignocellulose and starch conversions to desired products. Strategies that enhance thermostablity of enzymes both *in vivo* and *in vitro* are also assessed. Moreover, they made efforts to develop vectors which express recombinant enzymes in thermophilic hosts.

Kanekar et al. (1999) Studied on Bioremediation of phenol by alkaliphilic bacteria isolated from alkaline lake of Lonar, India and exhibited that Alkaliphilic bacteria were isolated from sediments of an alkaline lonar lake. They grow at pH 104 and phenol concentration of 500 mg/l. Alkaliphilic strains of *Arthrobacter* spp., Bacillus *cereus, Citrobacter ,fieundii, Micrococcus agilis* and *P.feudomonas putidu* biovar B were capable of removing phenol from waste waters arising from industries manufacturing methyl violet (using phenol as one of the major raw materials) and cumene-phenol. The waste waters from both these units were alkaline in nature (pH = 9.95-10.1) and had a high phenol content (368-660 mg/l). The alkaliphilic bacteria being studied removed 100% of the phenol from the industrial waste waters within 48 h of incubation under shake culture conditions and at an ambient temperature of 28 f 2 "C. Bioremediation of phenol by alkaliphilic strains of *Arthrobacter* spp., B. *cereus, C. freundii* and *M. ugilis* takes place

Tambekar and Tambekar (2013) studied on optimization of the production and partial characterization of an extracellular alkaline protease from thermo-halo-alkalophilic lonar lake bacteria and exhibited that the best carbon sources were fructose, maltose, starch and lactose respectively where as the best nitrogen sources were yeast extract, soy tone and soyabean cake respectively. While the most effective inorganic nitrogen sources was ammonium carbonate for *Bacillus pseudofirmus, Cohnella thermotolerans* and urea for *Bacillus odysseyi*. Supplementation of the culture medium with amino acid L-glutamic acid for *Bacillus pseudofirmus* and L-glycine for *Cohnella thermotolerans and Bacillus odysseyi* and metal ion Mg2+ for all the three *Bacillus* Species improved the protease production substantially. They produce alkaline protease at a maximum rate of optimum pH 10 and temperature at 75^{o}C.

Objectives:

- To isolate &characterize laccase producing extremophilic microbes (bacteria)

- To screen the isolated bacteria for production of laccase.
- To optimize production conditions of laccase.
- To study industrial applications of laccase on laboratory scale

Methodology:

Collection of sample from various extreme environments

Physiological, morphological characterization

Molecular characterization by analyzing 16s r RNA sequence,

Primary and secondary Screening of bacteria producing laccase

Production of laccase from screened isolates.

Purification of laccase by using proper method.

Optimization of physical and chemical factors affecting laccase.

Application of laccase.

Importance of work:

International status

Wang et al (2019) isolated Bacillus amyloliquefaciens and characterised laccase produced from it. And the recombined gene from bacteria is stable in extreme conditions like pH, high temperature, halides, heavy metals and detergents.

Shafiei et al (2019) isolated Cohnella sp. A01and studied laccase. Which is thermostable, detergent resistant, anti-environmental and industrial pollutants enzyme.its stability in extreme conditions made this enzyme important in biotechnological and industrial applications.

Claus (2004) studied structure reactions, distribution of laccase and told that this enzyme is involved in the pathogenicity, immunity and morphogenesis of organisms and in the metabolic turnover of complex organic substances such as lignin or humic matter.he also concluded that Phylogenetically, laccases are members of the multi-copper protein family including ascorbate oxidase, ceruloplasmin and bilirubin oxidase.

Neifar et al (2018) used alkali halotolerent bacterial enzyme laccase for Modeling and Optimization of Textile Dye Decolourization .his study shows that genus Pseudomonas is the best laccase-producing and dye-degrading strain.

Siroosi et al (2016) Purified and characterised an alkaline chloride-tolerant laccase from a halotolerant bacterium, Bacillus sp. strain WT isolated from Urmia lake .laccaseactivity toward typical laccase substrates: syringaldazine and 2, 2-azino-bis (3-ethylbenzothiazoline-6 sulfonate). The extracellular laccase (0.01 U mL−1) decolorized sulphonyl green BLE up to 97% at pH 7.0 after two hour incubation at 35◦C, without any addition of mediators.

National status

Chandra & chowdhary (2014) studied on applications of laccase for industrial waste bio remediation and concluded that this enzyme is efficient in degrading industrial pollutants enzyme's thermostability and enantioselectivity require attention for researcher.

Rajput & mishra (2018) studied on bio degradation of pyridine raffinate using bacterial laccase from garden soil. From this study they successfully isolated laccase producer ie pseudomonasfluorescens and concluded that biodegradation of effluent by this enzyme reduce C.O.D of the effluent.

Sharma et al (2006) studied on bacterial laccase and concluded that laccase enzyme is widespread in bacteria. He compared bacterial laccase with fungal laccase and their applications

Significance

- By this study we may be able to produce new industrially important laccase.
- This study may be able to produce previously discussed enzyme which will reduce its production cost.
- The produced laccase**s** may be more efficient than previous enzymes.

Proposed work plan:

First year:-

- Collection and isolation of extremophiles from particular habitat.
- Characterization of isolates by different methods.
- Screening of isolates to obtain laccase producers.

Second year:-

- Production of laccase from screened isolates.
- Study of factors affecting on enzyme.
- Study of enzyme activity.

Third year:-

- Characterization of enzymes.
- Purification of enzymes.
- Applications of enzymes produced from isolates.

Refrences:-

1 Sharma.p, Goel r, Capalash.n (2007): Bacterial laccases,World J Microbiol Biotechnol 23:823–832

2 Rajput.M.S, Mishra.B.N(2018):Biodegradation of Pyridine Raffinate using Bacterial Laccase isolated from Garden Soil, *Biocatalysis and Agricultural Biotechnology,S1878-8181(18)30758-8.*

3 *Wang .H,Huang .L,Li .Y,Ma .J,Wang .s,Zhang .Y,Ge .X,Wang .N,Lu .F,Liu .Y(2019):Characterization and application of a novel laccase derived from* Bacillus amyloliquefaciens,International Journal of Biological Macromolecules,BIOMAC-13898; No of Pages 9

4 Zhu .D, Wu .Q(2019):Industrial Enzymes,Comprehensive Biotechnology (Second Edition), Academic Press, 2011, Pages 3-13.

5 Shafiei .M,Afzali .F,Karkhane .A.A,Ebrahimi .S.M,Haghbeen .K,Aminzadeh .S(2019):Cohnella sp. A01 laccase: thermostable, detergent resistant, anti-environmental and industrial pollutants enzyme,Heliyon 5 (2019) e02543,2405-8440.

6 Claus Harald(2003):Laccases: structure, reactions, distribution,Micron 35 (2004) 93–96.

7 Neifar .M, Chouchane .H, Jdidi .A, Naili .F,Ouertani .R,Mahjoubi .M,Jaouani .A,Cherif .A(2018): Modeling and Optimization of Textile Dye Decolourization Using Laccase-Producing, Alkali-Halo tolerant Bacteria, International Journal of Textile Science & Engineering,TSE-107.

8 Chandra .R. C, Chowdhary .*P(2014):* Properties of Bacterial Laccases and Their Application for Bioremediation of Industrial Wastes,Environ. Sci.: Processes Impacts,1-40.

9 Siroosi .M, Amoozegar .M.A.,Khajeh .K(2016):Purification and characterization of an alkaline chloride-tolerantlaccase from a halotolerant bacterium*, Bacillus sp.* strain WT,Journal of Molecular Catalysis B: Enzymatic 134 (2016) 89–97

10 Thakker C.D. and Ranade: An alkalophilic *Methanosarcina* isolated from Lonar crater. *Current science* (2002) 82, 455-458.

11 *Benjamin M. Z, Matthew W.K, AndrewJ.L, ChristopherT.S , Michael W.W.Adams and Robert M.K:* Extremely thermophilic microorganisms as metabolic engineering platforms for production of fuels and industrial chemicals. *Frontiers in Microbiology* (2015).

12 P.P. Kanekar, S.S. Sarnaik and A.S. Kelkar: Bioremediation of phenol by alkaliphilic bacteria isolated from alkaline lake of Lonar, India. *Journal of Applied Microbiology Symposium Supplement (*1999), 85, 128s-133s.

13 C. Sa´nchez-Porro, S. Martı´n, E. Mellado and A. Ventos C. Sa´nchez-Porro, S. Martı´n, E. Mellado and A. Ventosa a: Diversity of moderately halophilic bacteria producing extracellular hydrolytic enzymes. *Journal of Applied Microbiology* (2003), 94, 295–300.

14 Tambekar S. D. And Tambekar D. H.: optimization of the production and partial characterization of an extracellular alkaline protease from thermo-halo-alkalophilic lonar lake bacteria, *Bioscience Discovery*. (2013), 4(1): 30-38.

15 Bergquist P. L., Morgan H. W. and Saul D.: Selected Enzymes from Extreme Thermophiles with Applications in Biotechnology. *Current Biotechnology, (*2014), *3,* 45-59.

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